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Chromosomal Amplification of the bla_{OXA-58} Carbapenemase Gene in a *Proteus mirabilis* Clinical Isolate

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ABSTRACT Horizontal gene transfer may occur between distantly related bacteria, thus leading to genetic plasticity and in some cases to acquisition of novel resistance traits. *Proteus mirabilis* is an enterobacterial species responsible for human infections that may express various acquired β-lactam resistance genes, including different classes of carbapenemase genes. Here we report a *Proteus mirabilis* clinical isolate (strain 1091) displaying resistance to penicillin, including temocillin, together with reduced susceptibility to carbapenems and susceptibility to expanded-spectrum cephalosporins. Using biochemical tests, significant carbapenem hydrolysis was detected in *P. mirabilis* 1091. Since PCR failed to detect acquired carbapenemase genes commonly found in *Enterobacteriaceae*, we used a whole-genome sequencing approach that revealed the presence of bla_{OXA-58} class D carbapenemase gene, so far identified only in *Acinetobacter* species. This gene was located on a 3.1-kb element coharboring a bla_{AmpC}-like gene. Remarkably, these two genes were bracketed by putative XerC-XerD binding sites and inserted at a XerC-XerD site located between the terminase-like small- and large-subunit genes of a bacteriophage. Increased expression of the two *bla* genes resulted from a 6-time tandem amplification of the element as revealed by Southern blotting. This is the first isolation of a clinical *P. mirabilis* strain producing OXA-58, a class D carbapenemase, and the first description of a XerC-XerD-dependent insertion of antibiotic resistance genes within a bacteriophage. This study revealed a new role for the XerC-XerD recombinase in bacteriophage biology.

KEYWORDS OXA-58, *Proteus mirabilis*, XerC-XerD recombinase, bacteriophage, carbapenems, *Acinetobacter baumannii*

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The transfer of antibiotic resistance genes (ARGs) across distantly related species contributes to the increasing antibiotic resistance observed in nosocomial and community-acquired human pathogens (1). ARGs may be acquired by conjugation, transformation, and transduction and may be carried by plasmids or inserted into the chromosome. Therefore, site-specific and homologous recombination plays a major role in the integration of ARGs either into mobile genetic elements (MGEs) or into the chromosomal backbone. The fixation of the insertion of an ARG is the result of a trade-off between the antibiotic resistance selection and the fitness cost of the insertion event (1). A great variety of mechanisms of insertion and of site specificity have been described (2). However, the role of phages in the dissemination is much less known than those of integrative and conjugative elements (ICE) and of plasmids.

TABLE 1 MICs[a] of β-lactams of *P. mirabilis* 1091, *P. mirabilis* CIP103181, *P. mirabilis* CIP103181 harboring plasmid pTOPO-OXA-58 or pTOPO-AmpC-like, *E. coli* Top 10, and *E. coli* Top 10 harboring plasmid pTOPO-OXA-58 or pTOPO-AmpC-like

Antimicrobial(s)	*P. mirabilis* 1091[b]	*P. mirabilis* CIP103181 (pTOPO-OXA-58)	*P. mirabilis* CIP103181 (pTOPO-AmpC-like)	*P. mirabilis* CIP103181	*E. coli* TOP10 (pTOPO-OXA-58)	*E. coli* TOP10 (pTOPO-AmpC-like)	*E. coli* TOP10
Amoxicillin	>256	>256	64	1	128	96	4
Amoxicillin + CLA[c]	>256	32	6	0.5	24	16	1
Ticarcillin	>256	>256	2	0.5	>256	6	1
Ticarcillin + CLA	>256	128	2	0.5	>256	6	1
Piperacillin	>256	>256	16	8	64	2	0.5
Piperacillin-tazobactam	>256	>256	8	8	8	0.5	0.5
Temocillin	128	16	1.5	1.5	64	8	8
Ceftazidime	0.046	0.125	0.064	0.064	0.125	0.25	0.125
Cefotaxime	0.032	0.016	0.032	0.032	0.047	0.125	0.125
Cefepime	0.094	ND[d]	ND	ND	ND	ND	ND
Aztreonam	<0.016	ND	ND	ND	ND	ND	ND
Imipenem	0.75	0.75	0.125	0.75	0.25	0.25	0.25
Meropenem	0.06	ND	ND	ND	ND	ND	ND
Ertapenem	0.125	0.064	0.006	0.064	0.012	0.006	0.006

[a]Values are in micrograms per milliliter.
[b]MICs for gentamicin, tobramycin, amikacin, ciprofloxacin, tigecycline, and colistin were >32, >32, 4, <0.06, 1, and >8 μg/ml, respectively.
[c]CLA, clavulanic acid at a fixed concentration of 4 μg/ml.
[d]ND, not determined.

Proteus mirabilis frequently causes urinary tract infections and bacteremia, usually related to indwelling catheters (3). Carbapenem resistance among *Proteus* spp. is mediated by the production of carbapenemases or through porin mutations with or without decreased expression of penicillin binding proteins (4). Carbapenemases involved in carbapenem resistance in *P. mirabilis* belong most frequently to either molecular class A (KPC-2) (5, 6), class B (VIM, NDM) (7, 8), or class D (OXA-48-like) (9). However, two *P. mirabilis* clinical isolates from France and Finland were reported to produce the carbapenemase OXA-23, which was exclusively found in *Acinetobacter* spp. (10). In addition, the production of acquired AmpC β-lactamases in *P. mirabilis* has been reported from Europe, the United States, and Asia (11).

OXA-58 is a widely spread carbapenem-hydrolyzing class D β-lactamase (CHDL) in imipenem-resistant *Acinetobacter* spp. (12). But it has also, on rare occasions, been described in *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Escherichia coli* in Sierra Leone (13), but no characterization of the genetic support was presented. In *Acinetobacter baumannii*, the bla_{OXA-58} gene is often plasmid borne but can also be chromosome borne (12, 14, 15).

Here we identified for the first time the bla_{OXA-58} gene in a *P. mirabilis* clinical isolate (*P. mirabilis* 1091). Analysis of the genetic context revealed an unusual association with a bla_{AmpC}-like gene and the integration of both genes by a likely XerC-XerD site-specific recombination event into an integrated prophage. Furthermore, we showed a 6-fold tandem amplification of the two-gene cluster leading to an increased expression. The characterization of this integration of two *bla* genes suggests a new role of prophages in the dissemination of ARG genes.

RESULTS AND DISCUSSION

Clinical case. A 53-year-old man was admitted at the UCL Namur University Hospital in May 2015 for surgical resection of an invasive colorectal adenocarcinoma and local radiotherapy. Two weeks later, the patient developed pyrexia at 39°C, with acute lower abdominal pain. *P. mirabilis* 1091, identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, was isolated from blood culture and subsequently from peritoneal fluid specimens. It was resistant to penicillin, gentamicin, tobramycin, and trimethoprim-sulfamethoxazole (besides natural resistance to colistin and tetracycline) and of reduced susceptibility to imipenem and ertapenem, while remaining susceptible to expanded-spectrum cephalosporins, aztreonam, fluoroquinolones, and tigecycline (Table 1). The patient was initially treated empirically with

piperacillin-tazobactam (4 g three times a day [TID]) and then switched to tigecycline (50 mg twice a day [BID]) and amikacin (1 g BID) following the antibiogram results. In view of the lack of clinical improvement despite apparently appropriate therapy based on laboratory results, radiographic and abdominal computed tomography (CT) scan imaging was performed, which revealed the presence of a voluminous (5- by 5-cm) pelvic abscess and a rectovesical fistula. Thereafter, the patient underwent a new surgical intervention with anterior resection of the rectum and formation of an end colostomy (Hartmann's proctosigmoidectomy). Following surgery, the patient gradually improved, and he was discharged home after 3 weeks.

***P. mirabilis* 1091 expressed a carbapenemase gene not previously reported in this species.** MICs of β-lactams for *P. mirabilis* 1091 confirmed its resistance to amino- and carboxypenicillins and its reduced susceptibility to carbapenems (Table 1), but the organism remained susceptible to expanded-spectrum cephalosporins according to EUCAST guidelines (http://www.eucast.org). High-level resistance to temocillin and piperacillin-tazobactam together with reduced susceptibility to ertapenem (MIC of 0.125 μg/ml and diameter inhibition zone size of 25 mm) at the threshold of the EUCAST screening cutoff for carbapenemase-producing *Enterobacteriaceae* (CPE) suggested the presence of a carbapenemase. Detection of carbapenemase-producing *P. mirabilis* isolates may be difficult based only on antibiotic susceptibility testing, since this species naturally displays reduced susceptibility to imipenem. Nevertheless, we have detected significant carbapenemase activity in *P. mirabilis* 1091 by two different biochemical tests: CARBA-NP and the recently described BYG test (16, 17) (data not shown). However, PCR failed to detect acquired carbapenemase genes commonly found in *Enterobacteriaceae* (data not shown). These negative PCR results suggested the presence in this strain of a carbapenemase gene not previously described in *P. mirabilis*.

Whole-genome sequence (WGS) of *P. mirabilis* 1091 revealed a bla_{OXA-58} gene. In order to characterize the resistome of strain 1091 and to identify a candidate gene responsible for its weak carbapenemase activity, we determined its complete genome sequence. The 5,087,888 matched 100-bp Illumina reads were assembled into 494 contigs (>200 bp in size), with a total length of 3,847,301 bp. The genome raw coverage was on average 132×. The antimicrobial resistome identified by using the Resfinder server (18) revealed the presence of the bla_{OXA-58} β-lactamase gene showing 100% amino acid sequence identity with OXA-58 from *A. baumannii* (15). This carbapenemase gene has never been reported in *Proteus*. Interestingly, RAST annotation (19) identified on the same 3.1-kb contig a second acquired β-lactamase gene showing significant sequence identity to bla_{AmpC} β-lactamase genes. The deduced AmpC protein was identical to an AmpC-like protein from an uncultured bacterium recovered in environmental samples in Peru (GenBank AMP47568) and 89% identical to AmpC from *Acinetobacter bohemicus* (GenBank WP_004650432) identified in soil from Czech republic (Fig. 1C) (20).

In agreement with the resistance phenotype of the strain, WGS analysis revealed additional acquired resistance genes to aminoglycosides (*strAB*, *aac*(*3*)-*IIa*, *aadA1*, and *aph*(3′)-*Ic*), chloramphenicol (*catB2*), tetracyclines [*tet*(J)], trimethoprim (*dfrA1*), and sulfonamides (*sul2*). Analysis of the genetic context of the contigs carrying these ARGs did not allow to determine unambiguously the localization of this gene except for *tet*(J), *aadA1*, *catB2*, and *dfrA1*, which are chromosome borne. The seven other genes are also possibly chromosome borne, given their average coverage by sequence reads and the absence of detected plasmids.

***bla_{OXA-58}* and *bla_{AmpC}* β-lactamase genes are expressed and functional in *P. mirabilis* and *E. coli*.** In order to study the expression of both *bla* genes, they have been amplified together with their promoter sequences, cloned into a pTOPO plasmid, and electroporated into *E. coli* TOP10 and *P. mirabilis* CIP103181. The β-lactamase OXA-58 expressed in *E. coli* TOP10 (pTOPO-OXA-58) and *P. mirabilis* (pTOPO-OXA-58) conferred resistance to amino-, carboxy-, and ureidopenicillins, which slightly decreased after clavulanic acid addition (Table 1). However, the MICs of imipenem and ertapenem were barely modified in the presence of the plasmid pTOPO-OXA-58. Similarly, the

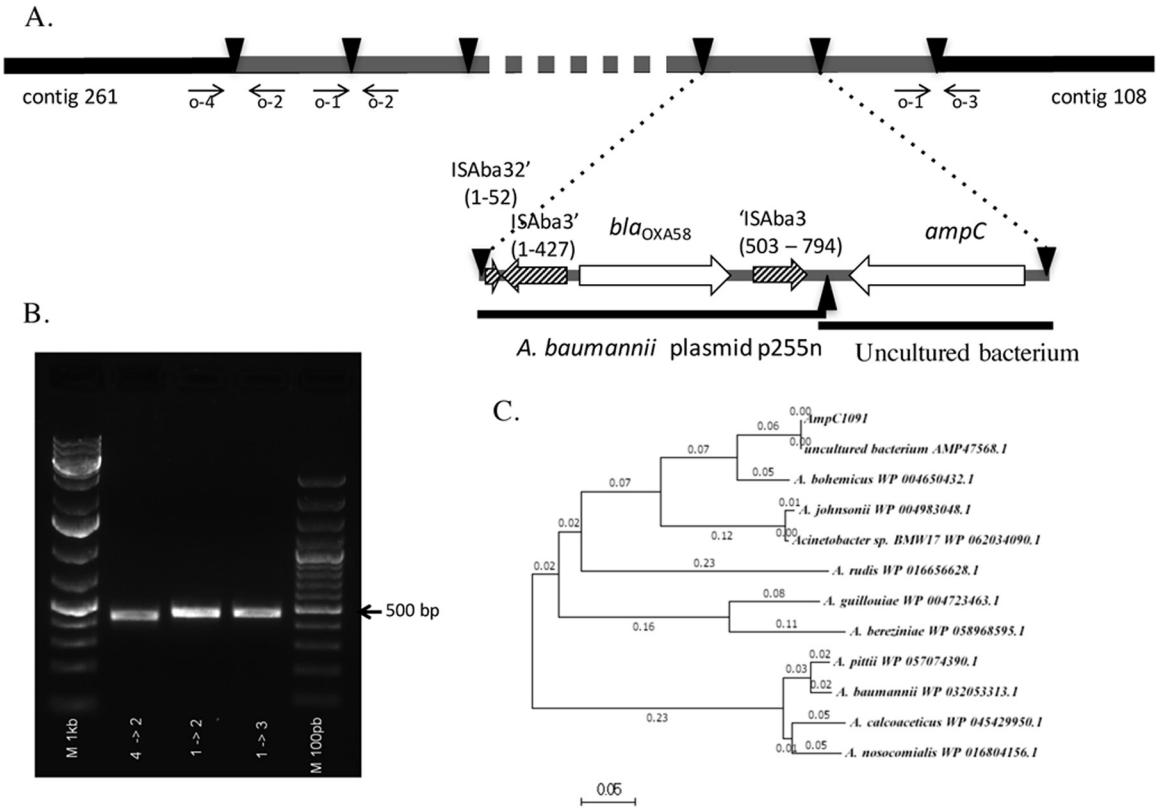

FIG 1 (A) Schematic representation of the chromosomal region encompassing the bla_{OXA-58} and bla_{AmpC} genes. The two β-lactamase genes and their orientations are represented by white arrows and partial ISs are represented by striped arrows. The dashed gray line in the upper panel represents additional tandem repeats of the bla_{OXA-58}-*ampC* locus. Regions of identities with an uncultured bacterium sequence CX_IN_B_Contig_19 and *A. baumannii* plasmid p255n are indicated in the lower panel. Black triangles represent the repeated sequence bracketing the tandem repeats and corresponding to partial putative XerC-XerD binding sites. The four oligonucleotides used to ascertain this chromosomal organization are represented by small arrows. (B) Picture of an ethidium bromide-stained agarose gel with the three PCR products. Numbers indicate the oligonucleotides used in the reaction. (C) Phylogenetic analysis of the new AmpC identified using a maximum likelihood method. GenBank accession numbers of the sequences used for this tree are indicated. The tree is drawn to scale with branch lengths measured in number of substitutions per site (next to the branches).

β-lactamase AmpC expressed by *E. coli* TOP10 (pTOPO-bla_{AmpC}) and *P. mirabilis* (pTOPO-bla_{AmpC}) conferred resistance to amino-penicillin, which only slightly decreased after clavulanic acid or tazobactam addition (Table 1). Our results suggest that both enzymes are expressed in *E. coli* and *P. mirabilis*, but likely at a low level, given the low MICs, especially for AMPC.

bla_{OXA-58} and bla_{AmpC} are tandemly amplified as an autonomous integrated element. The bla_{OXA-58} and bla_{AmpC} β-lactamase genes are located on a 3.1-kb contig showing a coverage six times higher than the rest of the genome (785× versus 132×). This high coverage suggested a plasmidic organization or tandem repetitions. Plasmid extraction using Kieser's method (21) from *P. mirabilis* 1091 did not reveal any visible plasmid, and repeated attempts to transfer the OXA-58 determinant to *E. coli* TOP10 by electroporation failed. These results, together with the absence of any plasmid-related sequences on this contig, suggested a chromosomal tandem amplification of the contig sequence. BLASTN comparisons of the 3.1-kb contig against the whole set of contigs revealed overlaps of 68 and 52 bases with one extremity of two contigs (108 and 261, respectively), suggesting tandemly repeated regions located between these contigs (Fig. 1A). To ascertain the chromosomal organization of the three contigs, we performed PCR experiments with primers located at the contigs' extremities: primers o-4 and o-2 for the junction between contig 68 and the 3.1-kb contig and primers o-1 and o-3 for contig 51 and the 3.1-kb contig (Fig. 1A). We also tested the structure of the tandem repetitions by PCR amplification with the divergent primers o-1 and o-2. The

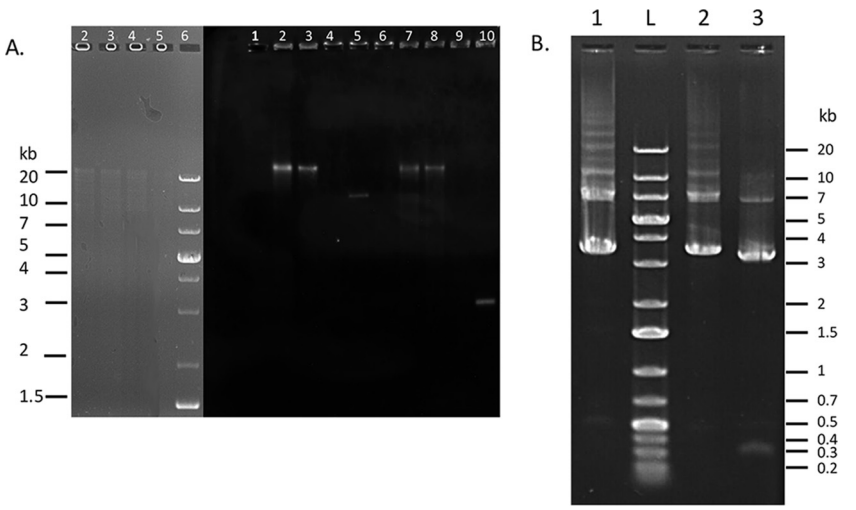

FIG 2 Southern hydribization of bla_{OXA-58} and LR-PCR amplification of the duplicated zone. (A) Southern experiments of *P. mirabilis* 1091 targeting the bla_{OXA-58} gene. Left, digest of total DNA; right, Southern hybridization. Lanes: 1, gene ruler DNA ladder 1kbPlus (ThermoFisher); 2 and 3, *P. mirabilis* 1091; 4, *P. mirabilis* CIP103181; 5, *A. baumannii* OXA-58; 6, ladder; 7 and 8, *P. mirabilis* 1091; 9, *P. mirabilis* CIP103181; 10, *A. baumannii* OXA-58. Lanes 2 to 5 correspond to the digestion by EcoRI, and lanes 7 to 10 correspond to the double digestion by BglII and SacII. Digestion of total DNA from an OXA-58-producing *A. baumannii* strain was included as a positive control of digestion and digestion of total DNA from *P. mirabilis* CIP103181 as a negative control. (B) LR-PCR amplification of the tandem duplicated bla_{OXA-58}, bla_{AmpC}-like region using primers o-4 and o-3 (Fig. 1). Lanes 1 and 2 correspond to the obtained amplicons, and lane 3 corresponds to the PCR products digested by XbaI, which cleaves inside the duplicated region.

three PCRs yielded products with the expected sizes, and subsequent Sanger sequencing confirmed the proposed genomic organization (Fig. 1B).

In order to confirm the proposed genomic organization, two sets of experiments were performed. First, Southern blot hybridization using EcoRI- or BglII/SacII-restricted *P. mirabilis* 1091 whole-genome DNA and a bla_{OXA-58} gene-specific probe was undertaken. For both digested genomic DNAs, a band of ca. 25 kb was observed (Fig. 2A). This size is in agreement with the expected sizes of 27.6 kb for the EcoRI digestion and 25.2 kb for the BglII/SacII double digestion, assuming, based on the coverage ratio, a six-tandem repetition of the bla_{OXA-58} and of bla_{AmpC} genes. Second, we used long-range PCR (LR-PCR) and primers o-3 and o-4 located on both sides of the repeated region to amplify it (Fig. 2B). A DNA ladder of five or six bands was observed. The smallest amplicon was ca. 3.5 kb in size, while the other fragments corresponded to multiples of about 3.2-kb fragments, with the largest band corresponding to ca. five or six copies (Fig. 2B, lanes 1 and 2). In other to further assess the specificity of the PCR products, an XbaI digestion that cuts within the duplicated fragment was performed (Fig. 2B, lane 3). After digestion, two fragments of expected sizes (ca. 300 bp and 3 kb) were obtained. A 7-kb band of unknown nature was also observed. Taken together, our results confirmed the hypothesized tandem-repeated 3.1-kb-long region of bla_{OXA-58}-bla_{AmpC} β-lactamase genes.

Integration and amplification of both bla_{OXA-58} and bla_{AmpC} gene sequences involved XerC-XerD binding sites. The insertion of the bla_{OXA-58} gene in *A. baumannii* plasmid occurred frequently at a XerC-XerD binding site (22). It has been assumed to be catalyzed by this chromosomally encoded recombinase responsible for chromosome dimer resolution (22). In order to reconstruct the origin of the bla_{OXA-58} and bla_{AmpC} fragments, we performed BLASTN analyses against the nr NCBI database and searched for putative XerC-XerD binding sites. The first 1,837 bp of the element are 99% identical to DNA regions from *A. baumannii* plasmids encompassing the bla_{OXA-58} gene and bracketed by two truncated IS*Aba3* sequences (Fig. 1A). We identified at both ends of these conserved regions sequences similar to the *P. mirabilis dif* site (the chromo-

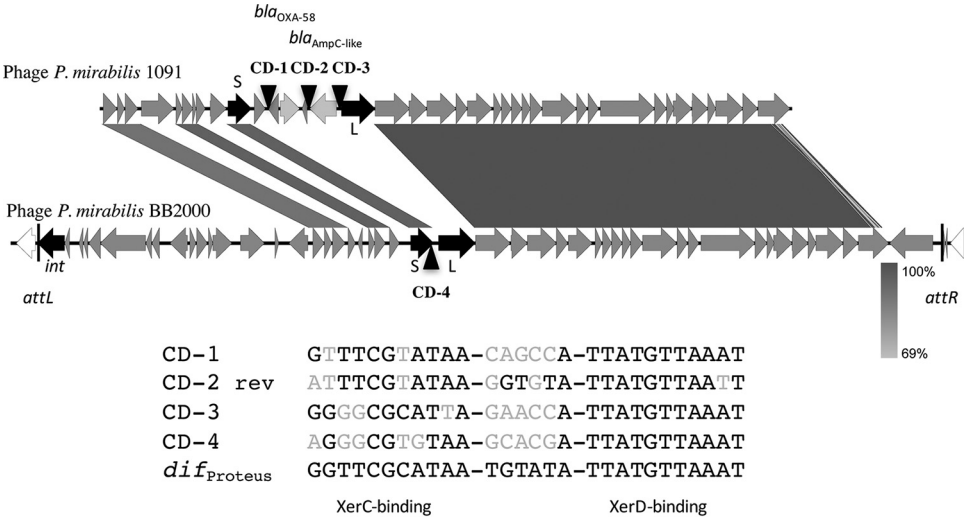

FIG 3 Prophage insertion of the bla_{OXA-58}-bla_{AmpC} element in *P. mirabilis* 1091. The bla_{OXA-58}-bla_{AmpC} element is inserted at a putative XerC-XerD binding site within a predicted prophage of *P. mirabilis* 1091. This recombination site is located in the intergenic region of two genes predicted to code for the small (S) and the large (L) subunits of the phage terminase, indicated by black arrows. (Top) Alignment of *P. mirabilis* 1091 partial phage sequence with the sequence of a closely related phage from strain BB2000 (GenBank CP004022). The two *bla* genes are indicated by light gray arrows, and the integrase gene of strain BB2000 prophage is indicated by a black arrow. The *attL* and *attR* sites are indicated by small vertical lines and the putative XerC-XerD binding sites by black triangles. Gray areas between open reading frames (ORFs) denote nucleotide identities with a gradient representing 69% (light gray) to 100% (dark gray) identity. (Bottom) Sequence alignment of the putative XerC-XerD binding sites with the predicted chromosomal *dif* site from *P. mirabilis* (33). The XerC binding sites on the left and XerD binding sites on the right are separated by six noncanonical bases. Shared nucleotides with the chromosomal *dif* site are indicated in black. CD-1, CD-2, and CD-3 are the predicted XerC-XerD binding sites bracketing the two *bla* genes in strain 1091; CD-4 was predicted in phage BB2000 between genes coding for the terminase subunits.

somal locus recognized by XerC-XerD) (23) (Fig. 3). The distal part of the element from bp 1804 to the end of the contig is 99% identical to the DNA sequence of an unculturable bacterium encompassing a bla_{AmpC}-like gene (Fig. 1A). This region overlaps by 33 bp the bla_{OXA-58} gene region and is bracketed by two XerC-XerD binding sites also conserved in the sequence of the unculturable bacterium (Fig. 1A and 3). Therefore, as described for the integration of bla_{OXA-58} gene into *Acinetobacter* sp. plasmids, the integration of both resistance genes occurred probably through a XerC-XerD recombinase-dependent mechanism. However, it is not possible to discriminate whether a successive insertion of each resistance gene into the progenitor of this strain occurred or whether both genes were inserted in a single step.

Tandem amplification in bacteria have been shown to frequently involve repeated elements, like insertion sequences (ISs) or rRNA operons (24). The duplication of the XerC-XerD binding site following the integration of the two genes yielded a 14-bp duplication at both ends of the sequence (Fig. 1A). This short repetition was probably sufficient for the first duplication event as previously reported (25). Subsequent amplifications may have occurred by replication slippage involving the duplicated 3.1-kb-long bla_{OXA-58} and bla_{AmpC} sequence. As the patient was treated by piperacillin-tazobactam before *P. mirabilis* 1091 was isolated, it is tempting to speculate that this treatment selected for the amplification of the two β-lactamase genes. However, we do not have access to isolates prior to this treatment to confirm this hypothesis.

The bla_{OXA-58} and bla_{AmpC} element is inserted into an integrated prophage. All reported cases of XerC-XerD-mediated insertion of antibiotic resistance genes showed that this insertion occurred at *dif*-like sites from large plasmids (26). These sites are recognized by XerC-XerD for the resolution of plasmid dimers. However, in *P. mirabilis* 1091 no plasmids or megaplasmids could be evidenced. In order to identify the genomic context of the XerC-XerD binding site, we performed an in-depth analysis of the region by automatic annotation and by BLASTN searches against published *P. mirabilis* genome sequences. It revealed that the two *bla* genes were inserted into a

prophage, partially assembled as a 30-kb contig. This partial prophage was 97% identical to a prophage from the completely sequenced *P. mirabilis* strain BB2000 (GenBank CP004022) (Fig. 3). The XerC-XerD binding site is located in a 2.2-kb region specific to the *P. mirabilis* 1091 prophage between two genes respectively encoding the phage putative terminase small and large subunits (Fig. 3). In the prophage of strain BB2000, the corresponding region encodes also the large subunit of a putative phage terminase (Fig. 3). The protein sequences of the two terminase large subunits were only 20% identical, a value in agreement with the absence of significant DNA sequence identities. Strikingly, we also identified a putative XerC-XerD binding site upstream from the terminase large subunit gene in strain BB2000 prophage (Fig. 3).

Besides its role in the resolution of chromosome dimers, the XerC-XerD recombinase has been shown to contribute to plasmid stability by resolving plasmid dimers at XerC-XerD binding sites (26). In addition, some integrative elements and phages devoid of an integrase gene like *Vibrio cholerae* phage CTXϕ, encoding the cholera toxin, exploit the XerC-XerD recombinase to integrate at the chromosomal *dif* site (22). These elements are more generally referred to as integrative mobile elements exploiting Xer (IMEXs). However, in the two *P. mirabilis* prophages that we analyzed, the XerC-XerD binding sites are not located at the extremities. In addition, strain BB2000 prophage is integrated at an *attB* site within the 5′ region of a tRNA-Pro gene and expresses an integrase gene. We conclude that in these phages, the XerC-XerD site is not involved in their integration. Furthermore, given their location beween two genes predicted to encode the two subunits of the phage terminase, it might be involved in the termination process in the course of the phage DNA encapsidation.

In *A. baumannii*, the $bla_{\text{OXA-24}}$ gene has been described as inserted at the XerC-XerD binding site of different plasmids, and it was suggested that following conjugation, the XerC-XerD recombinase catalyzed the transfer of the $bla_{\text{OXA-24}}$ gene from the plasmid of the donor strain to a resident plasmid of the recipient cell (27). Here, we propose that the integration of $bla_{\text{OXA-58}}$ and bla_{AmpC} genes into the chromosome of *P. mirabilis* 1091 results from the conjugative transfer of a likely *Acinetobacter* plasmid carrying the *bla* genes followed by a XerC-XerD recombination into the chromosomally integrated prophage. This recombination would be favored if the conjugative plasmid cannot replicate or is unstable in *P. mirabilis*.

Conclusion. This report represents the first isolation of a clinical *P. mirabilis* strain producing the OXA-58 carbapenemase. The $bla_{\text{OXA-58}}$ gene is widespread among carbapenem-resistant *Acinetobacter* species and is either chromosomally or plasmid borne (12). It is hypothesized that genetic exchanges occurred between an *Acinetobacter* species and *P. mirabilis* 1091, leading to the acquisition and expression of two β-lactamase genes, $bla_{\text{OXA-58}}$ and bla_{AmpC}, at the same chromosomal locus. The amplification of the inserted 3.1-kb fragment led to the observed phenotype. However, the number of repeats might increase under selective pressure. Since the strain has been cultured in the absence of selective pressure, the observed amplification (six repeats) might be underestimated here. *P. mirabilis* seems to be able to integrate CHDL from *A. baumannii*, but the extent of this is not known. Further studies are required to evaluate the prevalence of $bla_{\text{OXA-23}}$ or $bla_{\text{OXA-58}}$ genes in clinical *P. mirabilis* isolates. However, it is nearly impossible to suspect the presence of $bla_{\text{OXA-23}}$ or $bla_{\text{OXA-58}}$ genes based on MIC values of carbapenems only, which are barely above those of a wild-type (WT) *P. mirabilis* isolate. Only the combination of (i) reduced susceptibility to ertapenem and (ii) high-level resistance to temocillin and piperacillin-tazobactam might indicate the presence of a CHDL (classically OXA-48 but in rare cases OXA-23 or OXA-58). Since molecular methods developed for the detection of CPE do not include CHDLs from *Acinetobacter* spp., the use of a test able to detect a carbapenem-hydrolyzing activity (e.g., the Carba NP test and derivatives, the BYG test) followed by specific PCR is critical for the accurate detection of OXA-23- or OXA-58-producing *P. mirabilis* (28, 29) in complement to the already available multiplex PCRs targeting CHDLs from *Acinetobacter* species (30).

TABLE 2 Oligonucleotides used in this study

Name of primer	Sequence	Purpose
o-1	5′-CCATTCCTAACACGCCATA-3′	Structure of the bla_{OXA-58}-*ampC* region
o-2	5′-CTATGAAATTCAGCCTCAGC-3′	Structure of the bla_{OXA-58}-*ampC* region
o-3	5′-AACAAGTCGAAATTGACATCC-3′	Structure of the bla_{OXA-58}-*ampC* region
o-4	5′-GTGGGCGTCCTAAAGTACA-3′	Structure of the bla_{OXA-58}-*ampC* region
OXA-58-F	5′-ATACTCTCACTGAGGCAGGTTGG-3′	Cloning of the bla_{OXA-58} gene and bla_{OXA-58} probe
OXA-58-R	5′-CTGTCCCAATGATCACTTGCAA-3′	Cloning of the bla_{OXA-58} gene and bla_{OXA-58} probe
ampC-F	5′-TACTATGCTCAGCACAAGCC-3′	Cloning of the bla_{AmpC} gene
ampC-R	5′-TGGTGATGATATTGCTCTACG-3′	Cloning of the bla_{AmpC} gene

By analyzing the integration of the two β-lactamase genes into the chromosome of *P. mirabilis* 1091, we discovered the first case of a XerC-XerD-mediated insertion of an antibiotic resistance gene in a prophage. Integration into a prophage could represent a safe place, minimizing the fitness cost to the host cell. However, it is tempting to speculate that it may also allow an efficient way to disseminate ARGs by transduction. Furthermore, the presence of a XerC-XerD binding site in the vicinity of the putative terminase genes is a unique feature of this phage family.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and primers. A clinical *P. mirabilis* 1091 isolate was identified by MALDI-TOF mass spectrometry (MALDI Biotyper, Wissembourg, France). *P. mirabilis* CIP103181 and *E. coli* TOP10 (Life Technologies, Saint-Aubin, France) were used as hosts for electroporation experiments (8). The kanamycin-resistant pPCRBluntII-TOPO plasmid (Thermo Fisher Scientific, Cergy-Pontoise, France) was used as cloning vector. Bacterial cultures were grown in Trypticase soy (TS) broth at 37°C for 18 h. Primers used in this work are listed in Table 2.

Antimicrobial agents, susceptibility testing, and carbapenem-hydrolyzing activity confirmation. Antimicrobial susceptibilities were determined by the disc diffusion method on Mueller-Hinton (MH) agar (Bio-Rad, Marnes-La-Coquette, France) and were interpreted according to EUCAST guidelines (http://www.eucast.org). MICs were determined as recommended by the EUCAST using the Etest technique (bioMérieux, Marcy l'Etoile, France). The carbapenemase activity was assessed using the updated Carba NP test and the BYG test on colonies recovered from MH agar medium supplemented with $ZnSO_4$ as previously described (16, 17).

Nucleic acid extractions, PCR, whole-genome sequencing, and bioinformatic analysis. Total DNA was extracted from colonies using the Ultraclean Microbial DNA isolation kit (Mo Bio Laboratories, Ozyme, Saint-Quentin, France) according to the manufacturer's instructions. The DNA concentration was controlled by a Qubit 2.0 fluorometer using the double-stranded DNA (dsDNA) HS and/or BR assay kit (Life technologies), and the purity was estimated using Nanodrop 2000 (Thermo Fisher Scientific, Asnières, France). Frequently encountered acquired carbapenemase genes (bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{OXA-48}) in *Enterobacteriaceae* were sought by PCR using primers as previously described (31). The DNA library for WGS was prepared using the Nextera XT-v2 kit (Illumina, Paris, France) and then run on the HiSeq automated system (Illumina), using a 2 × 100-bp paired-end approach. *De novo* assembly was performed by CLC Genomics Workbench v7.0.4 (Qiagen, Les Ulis, France) after quality trimming (Qs [quality score] ≥ 20) with word size 34. The acquired antimicrobial resistance genes were identified using Resfinder server v2.1 (http://cge.cbs.dtu.dk/services/ResFinder-2.1/) (18). The genome was annotated using the RAST server (19). A phylogenetic tree was constructed using MEGA7 software (http://www.megasoftware.net/). The algorithm used for this purpose was the maximum likelihood method.

Investigation of the genetic context of the bla_{OXA-58}-bla_{AmpC} region was done by PCR using primers o-1 to o-4 (Table 2) as depicted in Fig. 1A. Q5 High-Fidelity DNA Polymerase was used according to the manufacturer's recommendations (New England Biolabs, Evry, France) for amplification of large fragments by long-range PCR (LR-PCR) using primers o-3 and o-4. Elongation time was set to 20 min, given the expected size of the fragment to amplify of ca. 20 to 25 kb (6 tandem repeats). Similarity searches were performed by BLASTN and BLASTP against the NCBI nr nucleotide and protein sequences databases, respectively. The search for XerC-XerD binding motifs was performed by using DNA strider (32).

Cloning of the bla_{AmpC}-like and bla_{OXA-58} genes into pTOPO. The bla_{AmpC}-like and bla_{OXA-58} genes were amplified by PCR with primers listed in Table 2 with Phusion polymerase (ThermoFisher Scientific) and cloned into pPCRBluntII-TOPO, as recommended by the manufacturer. Recombinant strains were selected on Trypticase soy agar (TSA) plates containing amoxicillin (30 μg/ml) and kanamycin (30 μg/ml).

Plasmid extraction and transformation. Hypothetical natural plasmids from *P. mirabilis* 1091 were extracted using Kieser's method and subsequently analyzed by electrophoresis on a 0.7% agarose gel as previously described (21), pTOPO derivatives were extracted using a GeneJET Plasmid miniprep kit (ThermoFisher Scientific, Illkirch, France) and introduced by electroporation into *E. coli* TOP10 and *P. mirabilis* CIP103181 using Gene Pulser II (Bio-Rad), and recombinant clones were selected on TSA supplemented with 50 μg/ml of ticarcillin (Sigma, St Quentin Fallavier, France).

Southern blotting and hybridization experiments. Briefly, genomic DNA of *P. mirabilis* 1091 was either digested with EcoRI or double digested with BglII/SacII restriction enzymes for 2 h at 37°C according to the manufacturer's recommendations (ThermoFisher Scientific, Illkirch, France). These enzymes were chosen because they do not cut within the 3.1-kb duplicated region. The DNA fragments were resolved on a 1% agarose gel prior to their transfer onto a Hybond-N^+ membrane (GE Healthcare, Fisher Scientific, France). Transferred membranes were probed with a PCR fragment specific for the bla_{OXA-58} gene (Table 2). The probe was labeled by using the ECL labeling kit according to the manufacturer's recommendations (GE health care, Orsay, France). Southern blotting was performed by *G:box chemi* using the manufacturer's recommendations (Syngene, Cambridge, UK).

Accession number(s). The genome of *P. mirabilis* 1091 has been deposited in the GenBank nucleotide database under accession numbers MCOR00000000 and KX668205.

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